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Axon Guidance: Morphogens Show the Way

Dispatch

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Hedgehog and Wnt family proteins can act as classic developmental morphogens to pattern a field of naïve cells. Surprising new studies show that members of these same protein families also act as guidance cues for growing axons in the developing nervous system.

The idea that molecular gradients instruct tissue differentiation is an old one. Two distinct modes of action for such graded signals have been postulated, experimentally demonstrated and molecularly characterised. First, a graded signal can pattern a tissue by instructing cells to select one of several alternative fates according to their position, as determined by measuring the absolute concentration of the signal. Such graded molecules are called morphogens, and they act by inducing transcriptional changes in the nucleus of the receiving cell [1,2]. Second, a graded signal can guide the migration of motile cells or cellular processes, such as axons. In this case, the cell or axon responds to the slope of the gradient rather than its absolute concentration. Such molecules are referred to as either chemoattractants or chemorepellents, depending on the direction of movement [3,4]. These guidance molecules act primarily by regulating cytoskeletal and membrane dynamics, not by signalling to the nucleus.

The long search for morphogens and guidance cues culminated in the identification of several highly conserved, but distinct protein families. Members of the Wnt, Hedgehog (Hh) and BMP families were shown to act as classical morphogens in a number of different contexts [1,2], while proteins of the Netrin, Slit and Semaphorin families were found to act as diffusible guidance cues for migrating cells and axons [4]. At first, it did not seem particularly remarkable that morphogens and guidance molecules should belong to distinct molecular families, given their very different modes of action. So it comes as something of a surprise to learn that morphogens and guidance cues may not be so different after all. Several recent studies have shown that members of each of the three classical morphogen families, Wnts, Hhs and BMPs, can also function as guidance cues [5–10]. In one particularly striking example [7], the same molecular gradient appears to be used both as a morphogen and as a guidance cue.

In asking whether a morphogen might act as a guidance molecule, there is one critical issue that has to be faced. Any manipulation of a morphogen gradient is expected to repattern the developing tissue, including any guidance cues within it. This may also lead to changes in the direction of cell or axon migration, but only indirectly. To demonstrate that a morphogen has a direct guidance function, one must show guidance can be altered without any change in cell fate — no simple task. Having a well-characterized system is the key.

One of the best understood systems for studying both pattern formation and axon guidance is the developing vertebrate spinal cord. Neuronal specification and axon guidance within the spinal cord are both controlled by signals emanating from two opposing sources: the dorsal roof plate and the ventral floor plate [11] (Figure 1A). Ventral fates are specified in response to a gradient of the morphogen Shh originating from the floor plate, and dorsal fates are specified by BMPs from the roof plate. Amongst the neurons specified in the dorsal spinal cord are the commissural neurons, which project axons ventrally towards and across the floor plate, guided in part by the chemoattractant netrin-1. The floor plate of netrin-1-deficient mice still attracts some commissural axons, however, implying the existence of at least one other floor plate attractant [12]. Earlier this year, Tessier-Lavigne and colleagues [7] identified this chemoattractant as none other than the floor plate morphogen Shh.

Using explant assays, Charron *et al.* [7] found that, like netrin-1, Shh also induces the attractive turning of commissural axons. Moreover, turning in response to either Shh-expressing COS cells or netrin-1-deficient floor plate (but not to netrin-1 or wild-type floor plate) was blocked by cyclopamine, an inhibitor of the Hh signal transducer Smoothened (Smo). This is strong evidence that Shh (or some other Hh protein) can account for most or all of the netrin-1-independent chemoattractant activity in the floor plate. But does Shh act directly on commissural axons, or indirectly by repatterning the ventral spinal cord?

There are three very good reasons to think it acts directly. First, the authors used an extensive set of markers to show that Shh does not repattern the spinal cord of embryonic day 11 (E11) embryos, as it does E10 explants, yet commissural axons in these E11 explants still turn towards Shh. Second, isolated *Xenopus* spinal neurons (which may or may not be commissural) turn towards Shh *in vitro*, a response that obviously cannot be explained by repatterning of surrounding tissue. Third, conditional inactivation of *smo* in commissural neurons does not change their fate, but leads to defects in axon growth through the ventral spinal cord. These findings make a compelling case that the same Shh gradient initially used as a morphogen to pattern the ventral spinal cord is later reused as a guidance cue that acts in concert with netrin-1 to attract commissural axons towards the floor plate (Figure 1B).

Just as Shh acts as both a morphogen and a guidance cue in the ventral spinal cord, members of another classical morphogen family, the BMP family, may control both patterning and guidance in the dorsal spinal cord (Figure 1). Thus, it seems that, amongst

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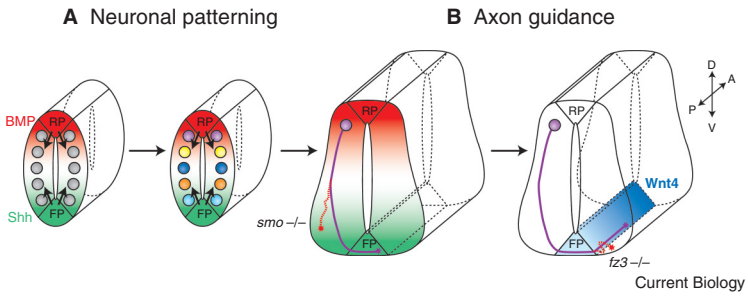

Figure 1. Morphogens as guidance cues for vertebrate commissural axons.

(A) Dorsal-ventral patterning of the spinal cord is controlled by BMPs (red) secreted by the roof plate (RP) and Shh (green) secreted by the floor plate (FP). Neurons select alternative cell fates (indicated by different colours) according to the local concentrations of these morphogens. (B) Later, these same molecular gradients are used as guidance cues to direct the growth of commissural axons (purple) towards and across the floor plate. For example, if commissural neurons lack Smoothened (*smo*$^{-/-}$), a mediator of Shh signalling, they follow aberrant trajectories in the ventral spinal cord (dashed red pathway). After crossing the floor plate, commissural axons are thought to be guided anteriorly along a gradient of Wnt4 activity in the floor plate (blue). In the absence of Frizzled3 (*fz3*$^{-/-}$), commissural axons often stall or turn posteriorly after crossing (dashed red pathway).

other cues, commissural axons are first 'pushed' away from the roof plate by one set of morphogens, the BMPs, then 'pulled' towards the floor plate by another, Shh. Evidently, these are not the only classical morphogens that guide commissural axons. Lyuksyutova *et al.* [6] present evidence that the task of guiding commissural axons may later be passed on to yet a third morphogen family, the Wnts.

Once commissural axons reach the floor plate, they extend across it and turn anteriorly, growing alongside the floor plate on the opposite side (Figure 1B). Zou *et al.* [13] had previously developed explant assays for the guidance responses of commissural axons after they have crossed the floor plate. Using these assays, they searched for tissues and candidate molecules that promote the anterior growth of post-crossing commissural axons. This search led to the Wnts, with Wnt4 emerging as the most promising candidate for an anterior attractant. The most dramatic demonstration of Wnt4's potent guidance effect is the ability of an ectopic Wnt4 source to redirect commissural axons posteriorly. As Wnt4 is expressed in an anterior to posterior gradient in the floor plate, it is well placed to provide a graded cue to guide axons anteriorly, at any rostrocaudal level.

Genetic evidence in support of this notion comes from the aberrant trajectories of commissural neurons in mice lacking the Wnt receptor Frizzled3. In these mice, the spinal cord appears to be patterned correctly, and commissural axons follow their normal trajectories towards and across the floor plate. But after crossing, many commissural axons either stall or turn posteriorly, as predicted for the loss of sensitivity to an anterior attractant. These are remarkable findings, but it is important to note that, while unlikely, an indirect role for Wnt4 cannot yet be excluded. Following the lead provided by the Shh studies [7], it will now be important to eliminate Frizzled3 function specifically in commissural neurons, and to ask whether Wnt4 can attract isolated axons *in vitro*.

Vertebrate Wnt4 is not the first Wnt protein to be implicated in axon guidance. Earlier this year, Thomas and colleagues [5] presented evidence that another Wnt, *Drosophila* Wnt5, also functions in axon guidance. Here too it involves the anterior versus posterior guidance of commissural axons, in this case before rather than after crossing the midline. But there are also other intriguing differences, as we shall see.

As in vertebrates, commissural axons in *Drosophila* also initially grow towards and across the midline. Axon guidance towards the midline is thought to involve chemoattraction by Netrins, as in vertebrates [14,15], but there is no evidence that either BMP or Hh proteins guide commissural axons in *Drosophila*. At the midline, these axons then choose between two alternative pathways across it: via the anterior commissure (AC) or posterior commissure (PC) of each segment (Figure 2). A few years ago, Thomas and colleagues [16] identified an atypical receptor tyrosine kinase called Derailed (Drl), and showed that it directs axons through the AC, evidently in response to a repulsive ligand localized along the PC. Drl is normally expressed on AC axons but is absent from PC axons. If PC axons are forced to express Drl they switch instead to the AC [16].

In the work reported earlier this year, Yoshikawa *et al.* [5] exploited this assay in a screen to find other genes required for Drl to transmit its repulsive guidance signal. One of the genes found in this screen encodes Wnt5. Not only is Wnt5 necessary for Drl to reroute PC axons through the AC, but, when ectopically expressed at the midline, Wnt5 is also sufficient to block the transit of AC axons that normally express Drl. These reciprocal epistasis experiments place Wnt5 firmly upstream of Drl in a pathway that controls axon crossing in the PC versus AC. Furthermore, Wnt5 also binds directly to Drl [5], presumably through Drl's extracellular WIF domain [17]. This supports a simple model in which Wnt5 itself is the repulsive ligand for Drl. It is important to note that, in all these genetic experiments, there is no evidence that loss or gain of *wnt5* or *drl* function in any way alters cell fates in the nerve cord. Evidently, Wnt5 acts directly in axon guidance through the Drl receptor.

How does the proposed role of Wnt5 in *Drosophila* commissural axon guidance compare to that of Wnt4 in vertebrates? Several major distinctions can be drawn. In *Drosophila*, Wnt5 is a highly localized and segmentally repeated cue, whereas in vertebrates Wnt4 appears to form a single gradient along the entire rostrocaudal axis. Another difference is that Wnt5 acts as a repellent for *Drosophila* commissural axons, whereas Wnt4 attracts vertebrate commissural axons. Such bifunctionality appears to be the rule rather than the

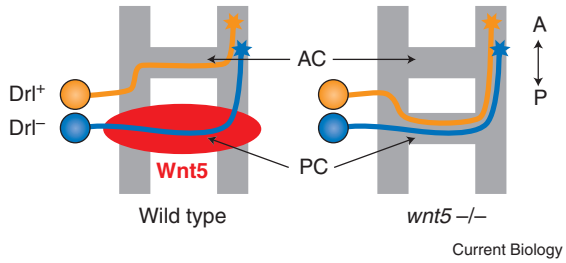

Figure 2. Wnt5 as a guidance cue for *Drosophila* commissural axons.

Commissural axons cross the midline in the anterior commissure (AC, orange axon) or posterior commissure (PC, blue axon). Wnt5 (red) produced by cells near the PC is thought to repel axons expressing the Drl receptor, forcing them to cross in the AC. In the absence of Wnt5 (*wnt5–/–*), some of these axons cross instead in the PC.

exception for guidance molecules. In some cases, the different growth cone responses to a single guidance cue have been attributed to the expression of different combinations of receptors [4]. This could also be the case for Wnts.

In *Drosophila*, Wnt5 signals through Drl, an atypical receptor tyrosine kinase, whereas in vertebrates Wnt4 appears to signal through the seven-transmembrane protein Frizzled3. It may simply be that Drl and Frizzled receptors act independently to signal repulsion or attraction, respectively; but it is also possible that Drl modulates signalling through Frizzled receptors. In such a model, Drl may not directly transduce a repulsive signal, but rather inhibit or reverse an attractive signal mediated by a Frizzled receptor, thus acting as an 'anti-attractant' rather than a *bona fide* repellent. Thomas and colleagues [5] do not favour this view, with good reason: they failed to observe any commissural defects in *frizzled* or *frizzled2* mutants, or to detect any genetic interactions between *frizzled* or *frizzled2* mutations and *drl*. But these are negative data, with the usual caveat that the failure to detect something is no proof that it does not occur. In this case, one important confounding factor is the existence of at least two other *frizzled* genes in *Drosophila*. The idea that Drl and other receptor tyrosine kinases may modulate Wnt signalling via Frizzled receptors is too appealling to be so readily dismissed.

With these new studies, axon guidance functions have now been documented for members of each of the three major families of classical morphogens: Hhs, BMPs and Wnts. This is exciting, not least because it was initially so unexpected. One of the obvious questions this work raises is how these molecules signal to the cytoskeleton to direct axon growth, rather than to the nucleus to specify cell fate. Another is how cells measure relative, rather than absolute, concentrations of these molecules, to extract directional rather than positional information from the gradient. Finally, it is worth considering the possibility that these 'morphogens' may even be the primary mediators of long-range guidance in the developing nervous system. Elegant genetic experiments, primarily in *Drosophila*, have provided compelling evidence that these molecules do indeed form gradients, and that they act directly and at a distance to influence cell fate [2]. Analogous experiments for guidance molecules such as Netrins and Slits are still lacking, so there is as yet no direct evidence that these molecules form gradients *in vivo* or that they signal directly and at a distance to steer growing axons. Such experiments are likely to be forthcoming, but until this is firmly established, we should at least be open to the idea that, *in vivo*, these molecules are mostly involved in short-range guidance, with much of the long-range guidance left to the specialists of long-distance signalling — the morphogens.

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